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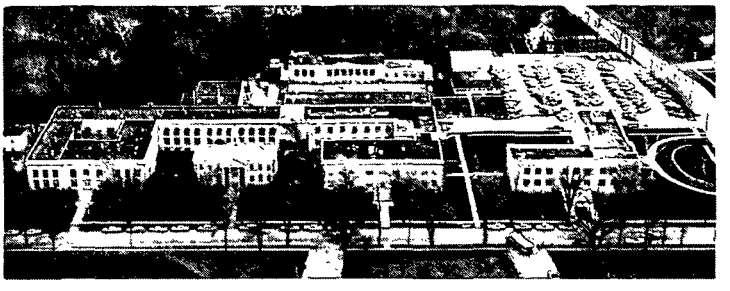

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EVALUATIONS OF A RAPID BACTERIAL TEST USING AN ATP ASSAY

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ABSTRACT

Paper and paperboard used in the packaging of certain foods is routinely monitored as to its bacterial content. The standard methods of assay impose a forty-eight hour delay before test results are known. The production flow during this period represents a substantial financial investment. It would, therefore, be to the manufacturer's advantage to become aware of a bacterial problem as soon as possible. A procedure was examined in which known amounts of contaminated milk carton stock were added to nutrient solutions and, after a defined growth period, the increase in cell number determined by means of a luciferin-luciferase assay for ATP. The results showed that the inherent dormancy periods and outgrowth rates of the Bacillus spores present in industrial board samples were too variable to provide a needed relationship between the initial bacterial count and the cell count after a period of growth. The rapid ATP assay, therefore, did not provide a reasonable estimate of the original board count.

INTRODUCTION

The U.S. Department of Health, Education and Welfare's sanitation standards dealing with single service containers for milk and milk products states that "Paper stock shall meet the bacteriological standard of not more than 250 colonies per gram as determined by the disintegration test"[1]. In order to measure compliance with this standard the manufacturers of milk carton stock routinely assay their product using one of several acceptable disintegration methods[2-4]. The methods are essentially alike and each requires an incubation period that imposes a forty-eight hour delay before the results are known. In the manufacture of milk carton stock the large machines produce a substantial quantity of board before a bacterial problem is recognized and corrective measures can be taken. Our prior experience with what is commonly known as the "firefly reaction"[5] suggested that it might serve to shorten the time interval between the initiation of a bacteriological test and the obtaining of the result.

Adenosine-5-triphosphate (ATP) is an essential ingredient in metabolic processes and, therefore, exists in all living organisms. Its reaction with the luciferin-luciferase components of firefly lanterns produces light in direct proportion to the concentration of ATP and has been used extensively to quantify living material in a wide variety of environments. The assay possesses exceptional sensitivity and routine procedures can detect as little as 10^{-6} µg of ATP, an amount permitting the detection of as few as 10^{4}-10^{5} bacteria per ml of fluid.[6] Starting with undetectable levels of bacteria growing in blood cultures, Gutekunst[7] found measurable levels could be obtained in as few as 5-6 hours although 12 hours were required for some of the slower growing gram positive types. The time required was also related to the original number of cells present in his materials. This prospect of a relationship between the original number of bacteria in a sample and their number after a period of growth in nutrient solution, combined with their detection via a sensitive ATP assay, provided the incentive for this attempt to develop a more rapid method to estimate the bacterial content of milk carton stock.

EXPERIMENTAL APPROACH

In a conventional disintegration test, such as the TAPPI method[2], a 1% slurry is prepared from a board sample and aliquots distributed into Petri dishes. A nutrient agar medium is added which solidifies about the organisms present in the sample. Incubation of the plates for a prescribed 48-hour period permits the organisms to multiply sufficiently to produce a visable colony of cells. Each colony is assumed to have originated from a cell initially present in the board sample and, therefore, the total number of colonies equals the original number of cells. It should be noted that it requires upwards of 10^{8} cells to produce a visable colony, a number that is significantly greater than that required for a positive response via ATP.

For the rapid assay a slurry is also prepared and treated with a nutrient of the same type and level as that used in the conventional assay with the exception that the agar solidifying agent is omitted. The organisms added with the fiber multiply and when their number reaches a level sufficient to be measured by means of their ATP content, they are assayed. The estimate of their original number at 0 time in this instance will depend on the consistency of the relationship of that number to the total cell number developing in the nutrient fortified fluid after a set period of time.

METHOD AND MATERIALS

The relationship between the initial bacterial

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count of a board sample and the ATP level after their outgrowth was explored in two phases. In the first phase, sterile fiber slurrys were inoculated with predetermined levels of spores of Bacillus subtilis var. niger (ATCC No. 9372). In the second phase, mill-produced milk carton stock samples containing various levels of bacterial contamination over the range of approximately 10-500/g were evaluated.

SLURRY PREPARATION

A slurry of 1% fiber content was prepared from 0.024-inch milk carton stock using standard disintegration procedures[2]. The board used in the inoculation studies as well as that used to prepare sterile controls in the second phase, was sterilized by autoclaving at 15 psi for 30 minutes before disintegration. A 50-ml volume of slurry was added to a 300-ml baffled shake flask (Bellco No. 599) and fortified by the addition of 50-ml of tryptone glucose extract broth (Difco No. 0750) that had been preheated to 50°C. The final nutrient concentration was 9 g/L and the fiber level was 0.5%.

INOCULATION (PHASE 1 ONLY)

A suspension of totally dormant Bacillus subtilis spores, prepared eight years ago and held in phosphate buffer (pH 7.2) at 5°C since that time, served as the inoculum source. The suspension was diluted in distilled water and appropriate amounts added to the sterile fiber slurrys to give spore levels of approximately 125, 250, 500 and 1000/g of fiber. Duplicate flasks were prepared for each spore level.

INCUBATION/OUTGROWTH

The baffled flasks were placed on an incubator shaker at 35°C and mixed at 130 cycles/minute. An incubation period of 10 hours was found sufficient for the Phase 1 inoculated flasks. Periods of 10, 12, 14 and 16 hours were examined in second phase using mill produced board samples. Flask preparation and assay times were staggered to maintain a constant outgrowth time for each individual flask.

CENTRIFUGATION

At the end of desired outgrowth period 15-ml volumes were removed from each flask and centrifuged lightly (170×g maximum) for one minute to partially settle the fiber. Subsamples for bacterial counts and ATP assay were drawn from the upper fiber-free portion of these samples.

ATP ASSAY

In general, the techniques described by Cheer, et al.[7] were followed with the following modifications. A 2-ml volume of centrifuged sample was added directly to 8 ml of boiling Tris buffer (pH 7.7). In order to control foaming caused by the nutrients present in the sample, 1 mg of Dow AF antifoam agent was added. After cooling and restoring evaporative losses with distilled water, the samples were either assayed promptly or frozen and stored at -20°C until convenient to assay.

The light output was measured on 0.1 ml of extracted sample mixed with 0.1 ml of rehydrated crude firefly lantern extract (Calbiochem B Grade) using an Aminco Chem-Glow photometer and integrator timer. The standard curve required to estimate the ATP level in the unknown samples was prepared by adding known amounts of ATP (Calbiochem No. 119191) to the Tris buffer and proceeding through the extraction steps using 2 ml of a centrifuged sample obtained from a sterile fiber control flask.

BACTERIAL EVALUATION

The original count of the board was determined by plating the fiber slurries as prescribed in the official TAPPI method[2]. A second count was made of each flask at each outgrowth sampling interval. Catalase tests[8] and microscopic examinations of stained preparations were carried out to confirm the presence of Bacillus species in all outgrowth positive flasks. Sterile materials and aseptic techniques were used throughout all aspects of study to prevent contamination by extraneous organisms.

RESULTS AND DISCUSSION

Prime considerations for method success were that the technique should: 1) be relatively easy to perform; 2) provide a substantial reduction in total test time and 3) demonstrate a consistent relationship between the level of ATP after outgrowth and the original count of the board sample.

ATP ASSAY PERFORMANCE

A number of the ATP assay procedures become quite involved due to a need to circumvent complications introduced via the substrates under investigation. Direct extraction in boiling Tris buffer is about the simplest of the procedures available to extract ATP from cells but is not usable if interferring substances are present in significant amounts in the cell-containing fluid being extracted. Initially, therefore, the presence of fiber, TGE broth and the antifoam agent in the outgrowth fluid required evaluation. It was found that each of these components did, in fact, reduce the light output of the ATP assay. The major interferring substance was fiber. An overall 88% drop in light output due to all components was reduced to 30% by eliminating most of the fiber from the extraction step. Light centrifugation adequately settled the large fibers without affecting the distribution of bacteria in the sample.

The remaining 30% loss in light output was accepted and incorporated into the experimental design by the addition of centrifuged fluid from the sterile control flasks to all standards used in the preparation of known ATP curves. A standard curve was prepared for each spore inoculation trial and each industrial board assayed. The expected straight line relationship was consistently obtained and the correlation between light output and total ATP remained very high ($r^2 = 0.9999$). In fact, the only significant difference observed between standard curves proved to be due to differing enzyme lots obtained from the manufacturer (Fig. 1).

SPORE INOCULUM PERFORMANCE

After the ATP assay evaluation indicated a satisfactory level of sensitivity could be achieved in a simple manner, the next phase involved the inoculation of sterile fiber slurrys with known levels of Bacillus subtilis spores. Many different bacterial types exist in and about the wet-end of a paper mill system, however, the heat applied in the drying of the board acts as a highly selective barrier. The result is that the surviving forms consist of the heat resistant spores of members of the genus Bacillus. Modelling of the test by means of Bacillus subtilis spores provided a greater degree of control over the initial count levels than could be obtained with real world board samples. Failure to meet test objectives in this phase would have quickly eliminated any hope of success using industrially produced board.

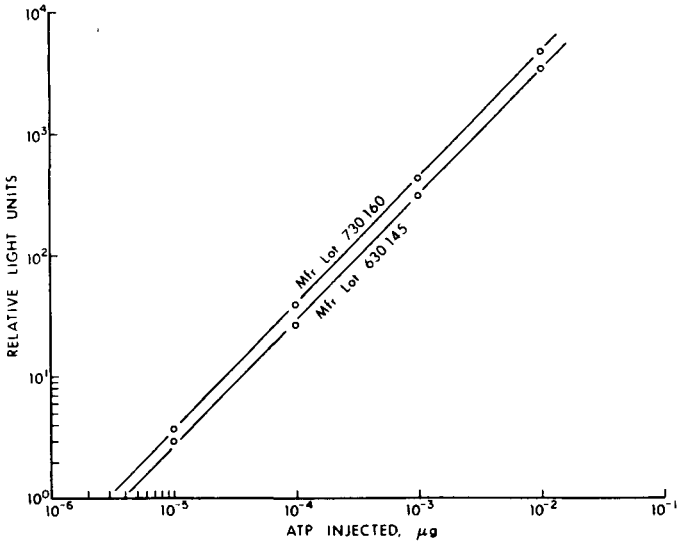

Figure 1. ATP Standard Curves

The optimum outgrowth period in the spore-inoculum trials was found to be 10 hours and the results of three such trials are given in Table I. The consistency of the outgrowth response between trials is readily evident. A high degree of correspondence was found for the relationship between the inoculated count and outgrowth ATP concentration, as well as outgrowth count and outgrowth ATP. The coefficients of determination were r^2= 0.93 and 0.99, respectively.

TABLE I

RESULTS OF INOCULATED TRIALS USING BACILLUS SUBTILIS VAR. NIGER SPORES

Inoculum, ct/g	10-Hr Count, cells/ml	10-Hr ATP, μg/ml	ATP/cell μg ($\times 10^{-9}$)
80	39,000	ND	--
360	44,000	ND	--
440	160,000	0.0016	10
1100	300,000	0.0022	7.3
160	46,000	ND	--
560	96,000	0.0013	14
640	200,000	0.0017	8.5
2400	640,000	0.0039	6.1
100	36,000	ND	--
360	79,000	0.0011	14
400	200,000	0.0017	8.5
1000	340,000	0.0027	7.9

ND - none detected.

The cell detection limit of the ATP assay was indicated to be near 50,000 cells/ml, however, this value was suspect as the ATP concentration per bacterial cell proved to be much higher than expected bacterial levels[9]. Since members of the genus Bacillus typically form chains of cells during growth, plate count methods commonly underestimate their true cell number. This fact would account for the high ATP values (Table I) found in this work. The real detection limit, therefore, is estimated to have been between 200,000 and 450,000 cells per ml of nutrient fluid.

COMMERCIAL BOARD SAMPLE PERFORMANCE

The final phase was the application of the foregoing nutrient outgrowth-ATP techniques to the analysis of ten samples of commercially produced board having a bacterial content ranging from 10 to 500 bacteria per gram. The correlation obtained for this phase between the initial board count and the outgrowth ATP showed a level of correspondence near that one might expect from a table of random numbers, i.e., r^2 = 0.05! The relationship between outgrowth count and outgrowth ATP also dropped (r^2 = 0.88) but remained sufficiently high to show that the difficulty did not lie in the ATP assay per se.

The failure to obtain the desired correspondence between the initial count and outgrowth ATP levels resulted from the fact that the species of Bacillus present in the industrial board samples varied widely in respect to their individual growth rates. Table II presents three examples of outgrowth patterns obtained with the industrial board samples. These clearly demonstrate the lack of a relationship between the initial sample count and the number of cells produced after a period of outgrowth in that the board sample containing 45 bacteria per gram produced a higher cell count after outgrowth than did the sample containing 490 bacteria/g. It was also apparent that one outgrowth period showed no advantage over another in this respect.

TABLE II

OUTGROWTH PATTERNS OBTAINED FOR PAPERBOARD SAMPLES OF VARYING BACTERIAL CONTENT

Outgrowth period, hr	Bacteria/ml 45/g	100/g	490/g
10	410,000	49,000	3,600
12	2,100,000	400,000	76,000
14	26,000,000	1,300,000	1,900,000
16	120,000,000	6,800,000	28,000,000

The underlying causes include the time required for the cells to break dormancy and initiate growth, as well as their subsequent dividing rate. The average cell dividing times, estimated from the industrial board samples, varied from 30 to 72 min, whereas in the laboratory model studies the dividing time was a consistent 38 ± 1.4 min for all trials.

CONCLUSIONS

The laboratory model using a spore inoculum indicated that a substantial reduction in total test time could be obtained by means of an ATP assay provided that the bacterial population was homogenous. At the outset it did not seem to be an unreasonable hope that with milk carton stock we would be dealing with a relatively homogenous population. Certainly a paper mill environment would not be suited to the growth of all the species of Bacillus and the heat of drying eliminates the other bacterial forms. However, results have proved otherwise and a needed relationship between the initial concentration of bacteria in a board sample and their number after a period of growth in nutrient solution did not exist. The application of an ATP assay to shorten the time required to obtain an estimate of the bacterial content of such board, therefore, was of no benefit.

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